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14. ABSTRACT
About one out of every ten cases of epithelial ovarian cancer (EOC) is inherited. The majority, >90%, of inherited cases of EOC are the result of mutations in the breast cancer associated gene 1 (BRCA1). This gene was originally identified based on genetic linkage to families with an increased risk of developing breast and ovarian cancer. It is involved in controlling normal cellular growth and is thought to suppress the growth of tumors. That is, if BRCA1 is mutated, the risk to develop breast and ovarian cancer increases. Another gene that is important in the development of cancer is p53. It also helps maintain normal cellular growth and is the most commonly mutated gene in all human cancers. The p53 gene has been shown to be mutated in at least 50% of all cases of epithelial ovarian cancer. In addition to mutations of BRCA1, mutations of the p53 gene are often found in patients with breast and ovarian cancer syndrome. Based on the importance of both of these genes in the development of this type of ovarian cancer, we hypothesize that inactivation of BRCA1 and p53 in the ovaries of mice will result in epithelial ovarian cancer in the animals.

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MODELING HUMAN EPITHELIAL OVARIAN CANCER IN MICE BY ALTERATION OF EXPRESSION OF THE *BRCA1* AND/OR *P53* GENES

PI: Denise C. Connolly
Progress Report, February 14, 2006

Introduction:

About one out of every ten cases of epithelial ovarian cancer is inherited. Unlike non-hereditary (sporadic) ovarian cancer, some of the underlying genetic causes of hereditary ovarian cancer are well understood. The majority, >90%, of inherited cases are the result of inherited mutations in the breast cancer associated gene 1 (*BRCA1*). This gene was originally identified based on genetic linkage to families with an increased risk of developing breast and ovarian cancer. It is involved in controlling normal cellular growth and is thought to suppress the growth of tumors. That is, if BRCA1 is mutated, the risk to develop breast and ovarian cancer increases. Another gene that is important in the development of cancer is the *p53* gene. It also helps maintain normal cellular growth and is the most commonly mutated gene in all human cancers. It has been shown to be mutated in at least 50% of all cases of epithelial ovarian cancer. In addition to mutations of *BRCA1*, mutations of the *p53* gene are often found in patients with breast and ovarian cancer syndrome. Based on the importance of both of these genes in the development of this type of ovarian cancer, we hypothesize that inactivation of *BRCA1* and *p53* in the ovaries of mice will result in epithelial ovarian cancer in the animals.

The objectives of this funded proposal are to:

1. develop mouse models of human epithelial ovarian cancer by inactivation of BRCA1 and p53 singly or at the same time in the mouse ovarian surface epithelial cells;
2. investigate whether there is a difference between the complete absence of p53 or the presence of a dominantly acting p53 mutant in ovarian tumorigenesis in mice; and,
3. identify genes and cellular pathways, downstream of BRCA1 and p53 inactivation/mutation, that contribute to ovarian carcinogenesis.

Body:

The approved Statement of Work for this proposal consisted of seven tasks for the 36 month funding period. Specific tasks that were proposed for months 12-24 of the funding period are included in tasks 2-7 and accomplishments, task by task, are reviewed below. Tasks outlined in original Statement of Work appear in italics. Work accomplished listed below each task in regular font.

Task 2. Generate, characterize, and establish breeding colonies of TgMISIIRrtTA-Cre transgenic mice (months 1-15).

a. Purify transgene construct from plasmid pMISIIR-rtTA-Cre.
b. Generate transgenic TgMISIIRrtTA-Cre mice (injection of embryos, implantation gestation).
c. Genotyping analysis of founder animals by PCR and Southern blot analysis.
d. Breed founders to generate F1 mice.
e. Genotyping analysis of F1 offspring.
f. Establish breeding colonies of five individual TgMISIIRrtTA-Cre transgenic lines of mice by crossing F1 offspring from each line.
g. Characterize offspring of individual TgMISIIRrtTA-Cre transgenic lines for expression of the Cre recombinase transgene following induction with systemic doxycycline by:
    - *Immunohistochemical detection of Cre recombinase in mouse tissues.*

- *Crosses of transgenic offspring with Rosa26R^{tm1Sor} reporter mice and detection of β-galactosidase expression in mouse tissues.*

h. Establishment and characterization of 5 transgenic lines, requires a total of approximately 80 mice.

At the time of submission of last year's progress report, seven Tg*MISIIRrtTA-Cre* transgenic lines were established and under characterization for Dox inducible expression of Cre recombinase in Tg*MISIIRrtTA-Cre*; Rosa26R^{tm1Sor} mice. The results from initial experiments assessing Dox-inducible expression of the β-galactosidase (β-gal) gene in three of seven transgenic lines were disappointing in that β-gal expression in the lines tested was weak at best. At the time of the 2005 progress report we had also demonstrated success in using intrabursal Adenovirus-Cre administration for the inactivation of floxed alleles. Although more technically challenging because of the requirement for survival surgery, we believe based on our results that this approach will allow temporal control and is likely to yield more robust Cre recombinase expression at the target tissue site. Based on this and in the interest of time we decided to use this method of Cre-mediated gene inactivation for all study groups. The adenoviral strategy will limit the number of triple genetic crosses required to obtain the necessary genotypes for each study group having the additional benefit of decreasing the time necessary to complete the studies.

Task 3. Adenovirus-Cre mediated inactivation loxP flanked (floxed) alleles of BRCA1$^{LoxP/LoxP}$, p53$^{LoxP/LoxP}$and BRCA1$^{LoxP/LoxP}$/p53$^{LoxP/LoxP}$mice (months 6-18)

a. Obtain sufficient numbers of animals that are homozygous for the floxed allele. Initially, 36 animals will be required for each strain evaluated.

b. Perform intrabursal injections on 30 female mice with recombinant adenovirus-Cre (Ad5-CMV-Cre) or 6 female mice with phosphate buffered saline as controls.

c. Monitor and evaluate animals for tumor formation, frequency and latency.

d. Histopathological evaluation of tissues of mice from each test group.

At the time of submission of the 2005 progress report, we completed Ad5-CMV-Cre injections on a total of 31 *BRCA1$^{LoxP/LoxP}$*mice ranging in age from 6-8 weeks (21 mice) 11 weeks (5 mice) and 14 weeks (5 mice). Over the past year, most of the Ad5-CMV-Cre injections that are planned for the entire project were completed and are summarized in Table 1. The Ad5-CMV-Cre injections are complete for *BRCA1$^{LoxP/LoxP}$and BRCA1$^{LoxP/LoxP}$/p53$^{LoxP/LoxP}$*mice. As stated previously, the *p53$^{LoxP/LoxP}$*mice are not robust breeders, therefore there has been a delay in completion of this group. The injections of the remaining ten *p53$^{LoxP/LoxP}$* mice (the remainder of task 3) are anticipated to be completed within the next month.

Table 1. Summary of total Ad5-CMV-Cre injections in mice bearing LoxP flanked alleles of *BRCA1* and/or *p53*.

	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Injection	**BRCA1$^{LoxP/LoxP}$**	**P53$^{LoxP/LoxP}$**	**BRCA1$^{LoxP/LoxP}$ P53$^{LoxP/LoxP}$**	**BRCA1$^{LoxP/LoxP}$ P53$^{WT/R172H}$**	**BRCA1$^{LoxP/LoxP}$ P53$^{LoxP/R172H}$**
Bilateral Ad5-CMV-Cre	**30 (34)***	**30 (26)**	**30 (41)**	**30 (34)**	**30 (0)**
Single ovary Ad5-CMV-Cre	**6 (6)**	**6 (6)**	**6 (6)**	**6 (5)**	**6 (0)**
PBS	**6 (6)**	**6 (4)**	**6 (6)**	**6 (0)**	**6 (0)**

*The number on the left indicates the target number of mice and the number in parentheses indicates the actual number of mice injected at the time of submission.

Mice that received Ad5-CMV-Cre injections are monitored twice a week for general health and well-being. In addition, the mice are palpated every other week to detect evidence of ovarian enlargement. A small number of mice were euthanized to demonstrate Cre-mediated excision of floxed alleles in the ovary or because they were moribund or had palpable tumors. The deaths are summarized in Table 2.

Table 2. Deaths and euthanasia in mice.

ID #	Age at death (days post Ad5-CMV-Cre)	Genotype	Reason for euthanasia or death	Necropsy performed	Tumor present	Tumor type	Excision
99	399 (301)	$BRCA1^{LoxP/LoxP}$	Moribund Abscess on shoulder	Yes	No	n.a.	BRCA1
100	189 (91)	$BRCA1^{LoxP/LoxP}$	Evaluate excision	Yes	No	n.a.	BRCA1
101	105 (7)	$BRCA1^{LoxP/LoxP}$	Evaluate excision	Yes	No	n.a.	BRCA1
149	236 (184)	$BRCA1^{LoxP/LoxP}$	Moribund Abscess on body wall	Yes	No	No	n.d.
298	90 (27)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Evaluate excision	Yes	No	n.a.	BRCA1 and p53
313	244 (181)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Evaluate excision	Yes	No	n.a.	BRCA1 and p53
344	222 (165)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Found dead	No	n.d	n.a.	n.d
314	239 (176)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Found dead	No	n.d	n.a.	n.d
345	291 (234)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Found dead	No	n.d	n.a.	n.d
334	325 (264)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Moribund	Yes	Yes	Osteosarcoma in peritoneal cavity, ovaries normal	Pending
307	352 (289)	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Palpable tumor	Yes	Yes	Apparent ovary tumor, awaiting histopathology	Pending
399	307 (250))	$BRCA1^{LoxP/LoxP}$ $p53^{LoxP/LoxP}$	Palpable tumor	Yes	Yes	Apparent ovary tumor, awaiting histopathology	Pending
412	151 (91)	$BRCA1^{LoxP/LoxP}$ $P53^{R172H/WT}$	Moribund	Yes	Yes	Lymphoma, ovaries appear normal	n.d.

n.d. = Not determined
n.a. = Not applicable

Excision of the appropriate floxed alleles was confirmed by PCR of genomic DNA isolated from short digestion of the ovary, thus confirming the activity of the Ad5-CMV-Cre recombinase on the target tissue. In recent weeks, three female $BRCA1^{LoxP/LoxP}$;$p53^{LoxP/LoxP}$ mice were euthanized and found to have tumors on necropsy. In one case (#334) the reproductive tract and ovaries appeared normal, but a solid tumor mass that was confirmed as an osteosarcoma was present throughout the peritoneum. In

two other cases (#307 and 399), large masses in the region of the ovary were palpated and on necropsy confirmed to involve the ovary. These masses were fixed in 10% neutral buffered formalin and are currently being sectioned and stained with hematoxylin and eosin (H&E). Additional sections will be stained for cytokeratins 8 and 19, vimentin, α-inhibin, and common leukocyte antigen (CLA) and sent to Dr. Alex Nikitin for complete histopathological evaluation. The remaining mice are currently being monitored twice a week with routine palpation to monitor ovary size and detect tumor masses.

Task 4. Obtain and establish breeding colonies of dominant-negative p53 ($p53^{R172H}$) mice (months 8-15).

- *a. Obtain breeding pair of $p53^{R172H}$ mice from Dr. Guillermina Lozano's laboratory (MD Anderson, Houston, TX).*
- *b. Establish breeding colony of these mice.*
- *c. Genotyping analysis by PCR and Southern blot analysis.*
- *d. Approximately 100 mice will need to be bred to obtain 36-40 female mice that are heterozygous for the mutated p53 allele.*

Task 4 was completed in year 1.

Task 5. Genetic crosses of TgMISIIRrtTA-Cre transgenic mice with $BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ mice and inactivation of conditional alleles by induction of Cre expression (months 15-30).

- *a. Cross TgMISIIRrtTA-Cre and $BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ mice.*
- *b. Genotype analysis of offspring by PCR and Southern blot analysis.*
- *c. Induce Cre recombinase expression in 30 TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ mice by systemic administration of doxycycline. A control group of 6 TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ mice will remain untreated.*
- *d. If necessary (i.e., if inactivation of BRCA1 or p53 alone leads to EOC), cross TgMISIIRrtTA-Cre and $BRCA1^{LoxP/LoxP}$ and TgMISIIRrtTA-Cre and $p53^{LoxP/LoxP}$ mice.*
- *e. If necessary, repeat task 5c. with TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ and TgMISIIRrtTA-Cre/$p53^{LoxP/LoxP}$ mice.*
- *f. Monitor and evaluate animals for tumor formation, frequency and latency.*
- *g. Histopathological evaluation of tissues of mice from each group.*
- *h. Generation of 36-40 TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ age matched female mice will require approximately 180-190 mice.*

As stated above, we obtained superior results using intrabursal injection of Ad5-CMV-Cre for delivery of Cre recombinase to the ovarian epithelium. Based on our findings, task 5 will not be performed. Instead, we injected both ovaries of 41 mice and single ovaries of 6 $BRCA1^{LoxP/LoxP}$/$p53^{LoxP/LoxP}$ mice with Ad5-CMV-Cre and both ovaries of 6 control mice were injected with PBS (see Table 1).

Task 6. Genetic crosses of $p53^{R172H}$ mice with TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ mice and induction of mutant p53 allele and inactivation of conditional BRCA1 allele by Cre expression (months 15-30).

- *a. Cross $p53^{R172H}$ and TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ mice.*
- *b. Genotype analysis of offspring by PCR and Southern blot analysis.*
- *c. Induce Cre recombinase expression in 30 $p53^{R172H}$/TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ mice by systemic administration of doxycycline. A control group of 6 $p53^{R172H}$/TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ mice will remain untreated.*

d. Monitor and evaluate animals for tumor formation, frequency and latency.
e. Histopathological evaluation of tissues of mice from each group.
f. Generation of 36-40 age matched female $p53^{R172H}$/TgMISIIRrtTA-Cre/$BRCA1^{LoxP/LoxP}$ mice will require a total of approximately 180-190 mice.

Because of the change in strategy favoring Ad5-CMV-Cre administration, no crosses will be made to Tg*MISIIRrtTA-Cre* transgenic mice. To develop strains containing floxed *BRCA1* and mutant $p53^{R172H}$, matings between $BRCA1^{LoxP/LoxP}$ and $p53^{R172H/R172H}$ mice were initiated and $BRCA1^{loxP/WT}/p53^{R172H/WT}$ offspring were obtained. The offspring were mated and female mice with the $BRCA1^{LoxP/LoxP}/p53^{R172H/WT}$ genotype were assigned to study group IV (Table 1) to receive Ad5-CMV-Cre or PBS injection. A total of 34 mice were injected bilaterally and 6 mice injected unilaterally with Ad5-CMV-Cre and we need to obtain 6 additional mice that will receive PBS injection as controls (Table 1). This group that will allow us to investigate the presence of both wild type and mutant (dominant negative) alleles of the p53 gene in the context of loss of BRCA1 gene function in the mouse ovary. Since we have elected to use the adenoviral approach, we decided to add an additional study group with the genotype $BRCA1^{LoxP/LoxP}/p53^{LoxP/R172H}$ that would allow us to mimic mutation of one allele of *p53* and loss of the second allele in combination with loss of *BRCA1* in the mouse ovary. We believe that this likely most closely reflects the situation that occurs in the context of human ovarian cancer with regard to *p53* status. To obtain these mice, mice with the genotype $BRCA1^{LoxP/LoxP}/p53^{R172H/R172H}$ are currently being mated to $BRCA1^{LoxP/LoxP}/p53^{LoxP/LoxP}$mice. All of the female offspring from these crosses will have the correct $BRCA1^{LoxP/LoxP}/p53^{LoxP/R172H}$ genotype and be assigned to group V for bilateral or unilateral injection of Ad5-CMV-Cre or PBS. This task should be complete with three to four months. One moribund $BRCA1^{LoxP/LoxP}/p53^{LoxP/R172H}$ mouse was euthanized and found on necropsy to have a large abdominal mass that was confirmed to be a lymphoma. This is not an unexpected result in that the $p53^{R172H/WT}$ mice do succumb to tumors of similar tissue spectrum (commonly lymphomas and osteosarcomas) and latency as $p53^{+/-}$ mice (1). Although mice in this study group may develop tumors outside of the ovary, we hope to observe lesions within the ovaries of mice in which *BRCA1* is inactivated that either outpace or occur concomitantly with other tumors.

Task 7. Identify differential gene expression by cDNA microarray analysis (months 18-36).
a. Isolate RNA from normal mouse ovarian surface epithelial cells and tumors.
b. Amplify reference (mouse universal RNA) and sample (normal and tumor samples) RNAs and prepare cDNA probes.
c. Hybridize microarrays.
d. Scan microarrays.
e. Analyze data, perform statistical analyses identify differentially expressed genes between normal and tumors and tumors that arise as a result of different combinations of genetic alterations.
f. Validate candidate genes by real-time PCR.

As tumors have been identified at this time, task 7 has not been initiated.

All mouse procedures described in this progress report were approved by Fox Chase Institutional Animal Care and Use Committee.

Key Research Accomplishments:

- Characterization of three of seven stable transgenic lines of Tg*MISIIR-rtTA-Cre* transgenic mice

- Intrabursal injection of 40 female *BRCA1*$^{LoxP/LoxP}$ mice with Ad5-CMV-Cre and 6 control mice with PBS
- Intrabursal injection of 32 female *p53*$^{LoxP/LoxP}$ mice with Ad5-CMV-Cre and 4 control mice with PBS
- Intrabursal injection of 47 female *BRCA1*$^{LoxP/LoxP}$/*p53*$^{LoxP/LoxP}$ mice with Ad5-CMV-Cre and 6 control mice with PBS
- Intrabursal injection of 41 female *BRCA1*$^{LoxP/LoxP}$/*p53*$^{R172H/WT}$ mice with Ad5-CMV-Cre
- Confirmation of Cre-mediated excision in Ad5-CMV-Cre injected ovaries
- Identification of ovarian tumors in two *BRCA1*$^{LoxP/LoxP}$/*p53*$^{LoxP/LoxP}$ mice injected with Ad5-CMV-Cre

Reportable Outcomes:

- Development seven lines of Tg*MISIIR-rtTA-Cre* transgenic mice and characterization of three of these lines
- Development of three new strains of mice *BRCA1*$^{LoxP/LoxP}$/*p53*$^{LoxP/LoxP}$, *BRCA1*$^{LoxP/LoxP}$/*p53*$^{R172H/WT}$ and *BRCA1*$^{LoxP/LoxP}$/*p53*$^{LoxP/R172H}$ mice
- Identification of ovarian tumors in two *BRCA1*$^{LoxP/LoxP}$/*p53*$^{LoxP/LoxP}$ mice injected with Ad5-CMV-Cre

Conclusions:

Based on the approved statement of work and the results obtained to date, this project is on track and at the expected stage. As we hypothesized, tumor formation is occurring in the ovaries of mice in which both *BRCA1* and *p53* were inactivated. We predicted that although it was possible that tumor formation might occur in mice in which only the *BRCA1* gene was inactivated in the ovary, that inactivation of both genes would lead to tumor formation with shorter latency. This result was observed in similar experiments in a mammary cancer model (2). Although very early in the study, preliminary results suggest that tumor formation appears to occur in mice with inactivation of both *BRCA1* and *p53* after relatively long latency.

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2. Xu, X., K. U. Wagner, D. Larson, Z. Weaver, C. Li, T. Ried, L. Hennighausen, A. Wynshaw-Boris, and C. X. Deng. 1999. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 22:37-43.

Appendices:

None